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NOTES

Concurrent Isolation of Chikungunya Virus and Dengue Virus from a Patient with Coinfection Resulting from a Trip to Singapore[▽]

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We report two cases of imported infection in patients who had returned to Taiwan from Singapore: one was coinfected with chikungunya virus and dengue virus type 2, and the other was infected with the same dengue virus. Both viruses were successfully isolated from the coinfected case by using antibody neutralization and a plaque purification technique.

Dengue fever, caused by a flavivirus in the family *Flaviviridae*, is the most prevalent arboviral disease in tropical and subtropical regions of Asia, the Pacific and Caribbean islands, and Central and South America (9). Chikungunya, caused by an alphavirus in the family *Togaviridae*, is endemic to Africa and Asia (12). Both diseases are transmitted to humans by day-biting *Aedes aegypti* and *Aedes albopictus* mosquitoes, and both diseases have similar clinical symptoms, including fever, rash, and joint pains as well as headache, fatigue, nausea, vomiting, and muscle pain; a laboratory test is required to distinguish between the two diseases. Thus, many risk factors for chikungunya virus (CHIKV) and dengue virus (DENV) infections are the same or similar. The urban mosquito *Aedes aegypti* is the primary vector of both viruses throughout most of their geographic range, although *Aedes albopictus* was recently identified as the main vector of the recently emerged CHIKV E1-226V variant of the African genotype (17).

The explosive epidemics of chikungunya in Indian Ocean islands and India since 2004 and the worldwide increase in travel have facilitated the expansion of different strains of CHIKV of the African genotype into overlap areas where DENV is endemic (13). As a result, cocirculation of CHIKV and DENV has been reported in various geographic areas, including India, Sri Lanka, Gabon, Cameroon, Madagascar, Malaysia, Indonesia, Singapore, and Thailand. Consequently, a few studies showing patients coinfected with CHIKV and DENV have been reported in India, Sri Lanka, Malaysia, and Gabon (1, 5, 8, 11, 14). Although molecular and serologic evidence demonstrated or suggested coinfections in the above-mentioned reports, neither CHIKV nor DENV was isolated from these patients. Successful isolation of both viruses is needed to conduct basic and applied research on CHIKV and DENV biology, immunology, and pathogenesis, as well as the development of laboratory diagnosis, antiviral drugs, and vaccines.

The first and only concurrent isolation of CHIKV and DENV-2, from a single blood specimen taken from a patient in the acute phase of a dengue-like illness in southern India in 1964, was reported by Myers and Carey (10). In their study, the dominance of CHIKV in the coinfected patient's serum, along with growth competition, prevented the initial isolation of DENV-2; isolation was finally accomplished through pretreatment of the acute-phase serum sample with a CHIKV-specific mouse antibody, followed by inoculation into infant mice for *in vivo* growth. Here we report only the second case confirmed by actual isolation of CHIKV and DENV-2, from a patient returned from Singapore, using an *in vitro* cell culture technique.

The two patients with cases of imported infection reported by our hospital surveillance system were part of a group tour to Singapore from 17 to 20 April 2009. One patient (case 1) was coinfected with CHIKV and DENV-2, and the other, a sibling of case 1 (case 2), was infected with the same DENV-2 strain. Table 1 shows the summary data from case 1, reported as a suspected dengue case on 23 April 2009. He had symptoms of fever, headache, vomiting, arthralgia, rash, and skin itch. Molecular screening for flavivirus and alphavirus infections using multiplex one-step SYBR green I-based real-time reverse transcription-PCR (RT-PCR) (15, 16) showed positive reactions to both alphavirus and DENV infections, suggesting the possibility of coinfection. Confirmation using specific primers showed positive reactions to CHIKV and DENV-2. The coinfection results were later confirmed by positive seroconversion of both CHIKV-specific and DENV-specific IgM and IgG antibodies in day 24 convalescent-phase serum samples. Case 2 had symptoms of fever, headache, muscle pain, and abdominal pain. The DENV-2 strain was successfully isolated from a day 4 acute-phase serum sample from case 2 by *in vitro* cell culture using the C6/36 cell line.

From the coinfected patient, CHIKV was readily isolated from the day 2 acute-phase serum sample by using the C6/36 cell line. However, initial isolation of DENV-2 was not successful, likely due to inferior growth competition with the dom-

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TABLE 1. Summary data from a patient (case 1) coinfected with CHIKV and DENV imported from Singapore

Parameter	Description or result
Patient	Case 1
Infections	CHIKV and DENV-2
Age (yr)	12
Gender	Male
Travel period in Singapore	17 to 20 April 2009
Onset of disease	22 April 2009
Clinical symptoms	Fever
	Headache
	Vomiting
	Arthralgia
	Rash
	Skin itch
Laboratory findings	
Real-time RT-PCR (day 2)	CHIKV$^+$, $10^{5.6}$ PFU/ml
	DENV-2$^+$, $10^{1.3}$ PFU/ml
Virus isolation (day 2)	CHIKV and DENV-2
DENV IgM/IgG (day 2)[a]	0.13/0.106
DENV IgM/IgG (day 24)	1.383/0.702
CHIKV IgM/IgG (day 2)	0.087/0.098
CHIKV IgM/IgG (day 24)	2.074/1.611

[a] Optical density at 405 nm, enzyme-linked immunosorbent assay.

inant CHIKV. To eliminate the CHIKV, neutralization was attempted by pretreatment of the acute-phase serum with a day 17 convalescent-phase serum from a CHIKV patient (15). This serum had high-titer CHIKV-specific antibodies but no DENV-specific antibodies. Briefly, the acute-phase serum from case 1 was mixed with CHIKV convalescent-phase serum at a ratio of 1:2 for 1 h at 37°C, and then the mixture was seeded in BHK-21 cells in a 6-well plate overlaid with methylcellulose prepared in minimal essential medium (MEM)-5% fetal bovine serum (FBS). The culture was incubated at 37°C for 5 days, and single plaques were picked for expansion in Vero cells. All 24 clones were DENV-2 isolates, as confirmed by an immunofluorescence test and RT-PCR (9).

The nucleotide sequences of the envelope genes of the CHIKV and DENV-2 strains were determined as previously described (6, 15, 16). Figure 1 shows the phylogenetic tree of CHIKV constructed on the basis of the complete envelope 1 gene nucleotide sequence (13). The strain CHIK/Singapore/0904aTw was grouped into the Central/East/South African genotype, with an E1-226V mutation, a lineage different from that of our previous Singapore isolate, with an E1-226A mutation, and is closely related to strains isolated from Malaysia. Figure 2 shows the phylogenetic tree of DENV-2 constructed on the basis of the complete envelope gene nucleotide sequences (18). The two isolates D2/Singapore/0904aTw and D2/Singapore/0904bTw, derived from case 1 and case 2, respectively, had 100% nucleotide identity in their envelope genes and belonged to the Cosmopolitan genotype. Our data are in agreement with recent reports that E1-226V mutant CHIKV strains grouped into the Central/East/South African genotype and DENV-2 strains grouped into the Cosmopolitan genotype were predominant epidemic strains circulating in Singapore in 2009 (7, 19). As various genotypic strains may differ in epidemic potential and virulence, molecular epidemiological surveillance can provide valuable information in decision making regarding patient care, outbreak investigation, and control measures (2, 4).

Successful isolation of both viruses from the coinfected case remains a challenge, since the dominant virus usually outgrows the minor virus. This problem can be overcome by a combination of virus neutralization using convalescent human serum with high-titer antibodies and *in vitro* plaque purification. Our results showed that this is a very efficient way to concurrently isolate both CHIKV and DENV-2. A similar approach has been used to isolate two DENV serotypes, DENV-1 and DENV-4, from a coinfected patient (3). With the expectation

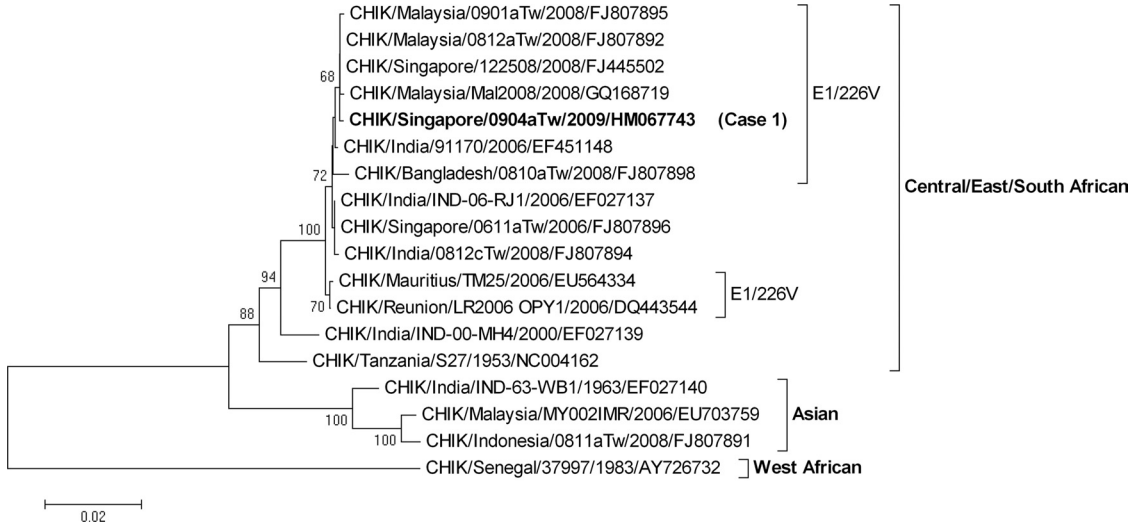

FIG. 1. Phylogenetic analysis of the complete envelope 1 (E1) gene (1,317 nucleotides [nt]) of a chikungunya virus (CHIKV) isolate from a patient who had returned from Singapore coinfected with CHIKV and dengue virus. The sequence obtained in this study is designated by boldface type. CHIKV strains with the E1-A226V mutation are indicated. Viruses are identified by virus/country/strain/year of isolation/GenBank accession number. The analysis was performed using MEGA 4 software, with the neighbor-joining (maximum-composite-likelihood) method. Bootstrap support values of >60 are shown (1,000 replicates). The scale bar on the left indicates the number of nucleotide substitutions per site.

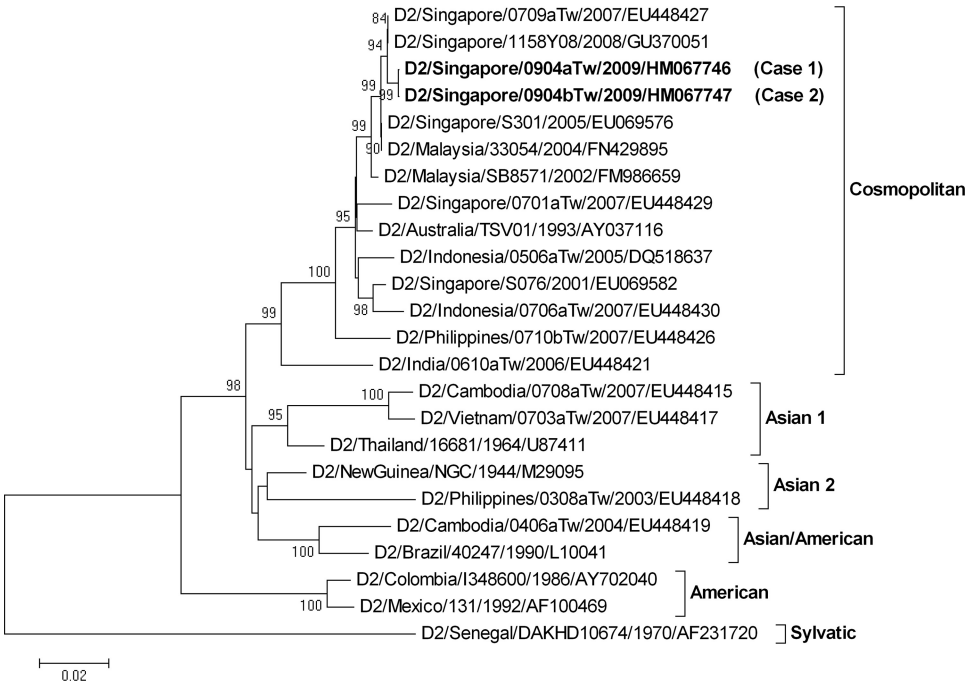

FIG. 2. Phylogenetic analysis of the complete envelope gene (1,485 nt) of dengue virus type 2 isolates from two patients (case 1 and case 2) who had returned from Singapore. Sequences obtained in this study are designated by boldface type. Viruses are identified by virus/country/strain/year of isolation/GenBank accession number. The analysis was performed using MEGA 4 software, with the neighbor-joining (maximum-composite-likelihood) method. Bootstrap support values of >75 are shown (1,000 replicates). The scale bar on the left indicates the number of nucleotide substitutions per site.

that cases of coinfection with DENV and CHIKV will become more prevalent in the future due to increased transmission of both viruses in various areas of India, Southeast Asia, and Africa, enhanced surveillance to clinically and diagnostically differentiate CHIKV and DENV infections is needed for early recognition of virus invasion and local transmission, better patient care, and timely control measures.

Nucleotide sequence accession numbers. The nucleotide sequences of the envelope genes of the CHIKV and the two DENV-2 strains were submitted to GenBank under accession no. HM067743, HM067746, and HM067747.

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